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Molecular Typing of *Staphylococcus aureus* Isolated From Clinical Specimens During an Eight-Year Period (2005 - 2012) in Tabriz, Iran

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Abstract

Background: Antibiotic resistant *Staphylococcus aureus* is a serious public health problem worldwide.
Objectives: This study aimed to investigate the susceptibility pattern and molecular typing of *S. aureus* isolated from clinical specimens of hospitalized patients during eight years, from 2005 to 2012.
Materials and Methods: A total of 151 randomly selected *S. aureus* isolates, identified with phenotypic tests and detection of *nuc* gene, were subjected to antimicrobial susceptibility testing using the disk diffusion method. Moreover, molecular typing of the isolates was carried out by PCR-RFLP based on *coa* and *spa* genes.
Results: All isolates were susceptible to vancomycin and teicoplanin. High rates of susceptibility were also observed with rifampin (98.1%), imipenem (94.7%), and linezolid (94.1%). On the other hand, most of the isolates were resistant against penicillin (95.4%), erythromycin (68.9%) and clindamycin (57.6%). Four types of *spa* and *coa* were distinguished among the isolates based on PCR results; however, the *HaeII* digestion resulted in a total of sixteen and nine RFLP patterns for *spa* and *coa* genes, respectively.
Conclusions: The outcome of this study indicates a higher discriminatory power of the RFLP analysis based on the *spa* gene compared to the *coa* gene. Moreover, the results of our study reveal that the resistance rate of *S. aureus* to some antimicrobial agents including linezolid is a growing concern.

Keywords: Antimicrobial Resistance, *Spa* Typing, *Coa* Typing, *Staphylococcus aureus*

1. Background

Staphylococcus aureus is one of the greatest concerns of all health-care-associated pathogens due to its ability to cause a wide variety of life-threatening infections including endocarditis, food poisoning, toxic shock syndrome, septicemia, skin and soft tissue infections as well as bone infections (1). In addition to the factors involved in the virulence of *S. aureus*, its resistance to antimicrobials contributes to its role as an effective opportunistic pathogen.

Methicillin resistant *S. aureus* was reported in 1961 from United Kingdom, shortly after methicillin's introduction in clinical practice (2). The rate of MRSA infections has increased dramatically since the mid-1980s (3). The surveys of the US association for professionals in infection control and epidemiology, Inc. (APIC) showed that the prevalence of MRSA in 2010 increased to 66.4 per 1000 inpatients compared to 46.3 in 2006 (4). The treatment options of MRSA are limited to few antibiotics like vancomycin, linezolid and tigecycline. Unfortunately, *S. aureus* isolates with decreased susceptibility to vancomycin (VISA) have recently been reported, which indicates that the data about antibiotic resistance in *S. aureus* isolates are critical for optimal decisions regarding infection control policies (5). *S. aureus* is a heterogenous species. Thus, in order to distinguish strains within this species for local epidemiologic or outbreak investigation purposes a highly discriminating genetic marker that accumulates variation rapidly is required (6). The pulse field gel electrophoresis (PFGE) is recognized as the most useful and discriminatory method for typing, but it is relatively difficult to standardize and is more time consuming than PCR-based methods since it requires culturing the bacteria (7). Alternatively, polymerase chain reaction (PCR)-based methods, targeting various genes such as protein A (*spa*) and coagulase (*coa*), can provide a rapid amplification, detection and typing tool for *S. aureus* strains (8, 9).

Nevertheless, there is a lack of data regarding *S. aureus* molecular types in Iran, particularly the northwestern

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part, as this could potentially result in transmission and establishment of undetected clones of *S. aureus*.

2. Objectives

The present study was conducted to perform the molecular characterization of *S. aureus* clinical isolates in northwest of Iran by evaluation of their antimicrobial susceptibility patterns in addition to molecular typing based on PCR-RFLP of *coa* and *spa* genes.

3. Materials and Methods

3.1. Sample Collection and Phenotypic Identification

In this study, the sample population consists of 151 isolates of *Staphylococcus aureus* which were selected randomly from stock ones isolated during eight years from 2005 to 2012 from various clinical specimens of patients admitted to the four teaching hospitals (Imam Reza, Sina, Shahid Madani and Kodakan) in Tabriz, northwest region of Iran. The isolates were identified as *S. aureus* based on bacterial growth on mannitol salt agar, colony morphology, gram staining, catalase, slide or tube coagulase and DNase tests (10).

3.2. Antibiotic Susceptibility Test

Antimicrobial profiling was performed by the disk diffusion method. The selection of an antibiotic panel for susceptibility testing is based on clinical and laboratory standards institute guideline (11). All antibiotic discs including penicillin (10 unit), oxacillin (1 μg), vancomycin (30 μg), teicoplanin (30 μg), gentamicin (10 μg), rifampin (5 μg), azithromycin (15 μg), erythromycin (15 μg), clindamycin (15 μg), ceftriaxone (30 μg), ciprofloxacin (5 μg), ofloxacin (5 μg), cotrimoxazole (25 μg), meropenem (10 μg), imipenem (10 μg) and linezolid (30 μg) were prepared from MAST company (Mast diagnostics, UK). *Staphylococcus aureus* ATCC 29213 was used as a control strain for the susceptibility testing.

3.3. Molecular Speciation and Detection of mecA

All isolates were confirmed as *S. aureus* by screening for the nuclease-encoding gene (*nuc*) and for methicillin resistance by *mecA* gene using a multiplex PCR as described previously (12). Chromosomal DNA was extracted using SDS-proteinase K with the CTAB method as prescribed by Sambrook et al. (13). The *S. aureus* ATCC 25923 and *S. aureus* ATCC 33591 strains were used as negative and positive controls for *mecA* and *nuc* genes, respectively.

3.4. Polymerase Chain Reaction-RFLP for spa and coa Typing

Based on the published sequences for the spa and the *coa* genes, the multiplex PCR was applied for amplification of target genes with the following primers: SPA1, 5'-ATC TGG TGG CGT AAC ACC TG-3' and SPA2, 5'-CGC TGC ACC TAA CGC TAA TG-3' (14), COA1:5'-CGA GAC CAA GAT TCA ACA AG-3' and COA2:5'-AAA GAA AAC CAC TCA CAT CAG T-3' (15).

The PCR master mix consisted of 1X PCR buffer, 1 mM MgCl2, 0.2 mM dNTPs (TAKARA, Japan), 1 unit of Taq DNA polymerase (TAKARA, Japan), 1 μM of primers and 5 μL of DNA extract in a final volume of 50 μL.

The PCR conditions were as follows: Initial denaturation at 94°C for 7 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute and extension at 72°C for 3 minutes with a final extension at 72°C for 5 minutes.

After amplification of the variable region of *spa* and *coa*, 10 μL of each amplicon was mixed and digested with 1 μL of *HaeII* restriction enzyme (MBI, Fermentas, Lithuania) at 37°C for 3 hours, and fragments were detected by electrophoresis in 1.5% agarose gels and subsequent ethidium bromide staining.

3.5. Statistical Analysis

The data were analyzed using the chi-square test with SPSS software version 22.0 (SPSS Inc., Chicago, Illinois, USA). A statistically significant difference was considered as a $P < 0.05$.

4. Results

Out of 151 *S. aureus* identified on the basis of phenotypic tests, all strains were positive for the presence of *nuc* gene, while *mecA* gene was detected in 54 (35.7%) isolates (considered as MRSA), and the remaining 97 (64.3%) isolates were identified as methicillin sensitive (MSSA).

Concerning the origin of isolates, Most of the strains [n = 62 (41.1%)] were isolated from wound, followed by blood culture [52 (34.4%)], urine [16 (10.6%)] and the remaining were obtained from specimens like synovial fluid, sputum, intravenous catheter and endotracheal tube.

4.1. Antimicrobial Susceptibility

According to disk diffusion assay, all isolates were uniformly found susceptible to vancomycin and teicoplanin, while few of them showed nonsusceptibility to rifampin (1.9%), imipenem (5.3%), and linezolid (5.9%). However, 95.4% resistance rate was observed to penicillin followed by 68.9% to erythromycin and 57.6% to clindamycin. Table 1 shows the antimicrobial resistance pattern of tested isolates.

Table 1. Antimicrobial Susceptibility Pattern of Tested *Staphylococcus aureus* Isolates

Antibiotics	Resistant Isolates	Intermediate Resistant Isolates	Sensitive Isolates
Penicillin	144 (95.4)	-	7 (4.6)
Oxacillin	52 (34.4)	-	99 (65.6)
Vancomycin	-	-	151 (100)
Teicoplanin	-	-	151 (100)
Gentamicin	28 (18.5)	5 (3.3)	118 (78.1)
Rifampin	3 (1.9)	-	148 (98)
Azithromycin	37 (24.5)	6 (3.9)	108 (71.5)
Erythromycin	104 (68.9)	15 (9.9)	32 (21.2)
Clindamycin	87 (57.6)	17 (11.3)	47 (31.1)
Ceftriaxone	36 (23.8)	-	115 (76.5)
Ciprofloxacin	16 (10.6)	21 (13.9)	114 (75.5)
Ofloxacin	12 (7.9)	2 (1.3)	137 (90.7)
Cotrimoxazole	31 (20.5)	-	120 (79.5)
Meropenem	8 (5.3)	-	143 (94.7)
Imipenem	9 (5.9)	2 (1.3)	140 (92.7)
Linezolid	9 (5.9)	-	142 (94)

4.2. Spa and coa Typing

The lengths of *spa* bands in the isolated bacteria were varied from 1000 to 1450 bp. These patterns were classified as type S1 (1450 bp), S2 (1250 bp), S3 (1100 bp) and S4 (1000 bp) including 36.4%, 32.4%, 23.2% and 8%, respectively. Moreover, *spa* amplicons, after digestion with *HaeII* restriction enzyme, showed distinct spa banding patterns. The restriction patterns of *spa* gene are shown in Table 2.

Regarding *coa* typing of all isolates, four distinct types were defined. These types were designated as C1 - C4 with fragments ranged from 500 to 900 bp. As shown in Table 2*HaeII* digestion of these PCR products yielded two (in the cases of C1, C2 and C4 types) or 3 (in C3 type) different restriction profiles.

5. Discussion

Staphylococcus aureus has always been a stumbling block for antimicrobial chemotherapy and the introduction of new classes of antimicrobial agents is usually followed by the emergence of resistant forms of this pathogen (16). Moreover, infections caused by *S. aureus* have a poorer prognosis when the infecting strain is MRSA (17). A lot of studies in developing countries demonstrate a continuing increase in MRSA infections (18, 19). The increasing incidence of MRSA infections most likely reflects the growing impact of medical interventions, devices, as well as antibiotic overusing, older age and comorbidities of patients (20). The prevalence of MRSA in the present study was 35.7%, which is comparable to that found by Fatholahzadeh et al. in Iran and Dar et al. in India (21, 22). However, this rate is less than half of the percentage reported in the other studies from Iran (23, 24). This observed difference could be attributed to the period of our study that was longer than others. Moreover, concerning the isolation time of bacteria in the current study, beginning since 2003, and considering the growing rates of MRSA over the years, the fairly low percentage of MRSA in our study is justifiable.

In the present study, according to PCR and disk diffusion results, we have detected two *S. aureus* isolates positive for *mecA* gene but susceptible to oxacillin disk. The occurrence of these variants could be explained by the presence of complete regulator genes (*mecI* and/or *mecRI*), as described previously (25). Only a low proportion of isolates in our study presented susceptibility to penicillin. It was expected, since, currently, it has been recognized that only a small percentage of *S. aureus* clinical isolates are not β-lactamase producer (26). Linezolid resistance shown by 5.9% of *S. aureus* isolates in the present study is one of the significant and clinical relevant observations, as there are several studies from Iran and other countries reported that almost all of clinical strains of *S. aureus* still remained susceptible to linezolid (27-30). Indeed, linezolid is the first representative of a new synthetic class of antibacterial oxazolidinones, which inhibits bacterial protein synthesis in a different mode from that of other protein synthetic inhibitors at the chain elongation step (31). Researchers assumed that resistance to linezolid would never develop. However, linezolid-resistant *S. aureus* appeared within 1 year after linezolid was approved for therapeutic use (32).

In agreement to most earlier reports (21, 27, 33, 34) vancomycin and teicoplanin resistance were not observed among our isolates which indicate that vancomycin is still the drug of choice for the treatment of life-threatening infections of *S. aureus*, although recently isolation of vancomycin resistant *S. aureus* from some countries has confirmed that emergence of these strains is a global issue (34, 35). Furthermore, it should be noted that the disk diffusion agar test did not accurately identify resistance to vancomycin in *S. aureus* and broth or agar dilution methods or E-test are needed (36).

Understanding the molecular characteristics of *S. aureus* isolates is important for assessing the relatedness of isolates, and consequently, for the implementation of appropriate infection control measures (37).

The *spa* and *coa* genes in *S. aureus* isolates have various numbers of degenerate repeats, which are clearly poly-

Table 2. Pattern of *spa* and *coa* Genes Diversity Among *Staphylococcus aureus* Isolates

Types	PCR Amplicon Size, bp	No. (%)	RFLP Pattern, bp	No. (%)
S1	1450	55 (36.4%)		
S1a			250, 1200	5 (3.3)
S1b			250, 500, 650	35 (23.2)
S1c			300, 1100	4 (2.6)
S1d			200, 500, 750	2 (1.3)
S1e			600, 800	6 (4)
S1f			150, 500, 800	3 (2)
S2	1250	49 (32.4%)		
S2a			350, 900	20 (13.2)
S2b			400, 800	15 (9.9)
S2c			1250	14 (9.3)
S3	1100	35 (23.2)		
S3a			200, 300, 600	9 (6)
S3b			400, 700	3 (2)
S3c			600, 500	22 (14.6)
S3d			300, 750	1 (1)
S4	1000	12 (8)		
S4a			350, 600	3 (2)
S4b			300, 700	3 (2)
S4c			450, 500	6 (4)
C1	500	46 (31.5%)		
C1a			500	22 (14.5)
C1b			400,100	13 (8.5)
C2	600	19 (12.6)		
C2a			200, 400	14 (9)
C2b			600	5 (3)
C3	700	53 (35)		
C3a			250, 350	13 (8.5)
C3b			200, 300,180	24 (16)
C3c			700	17 (11)
C4	900	33 (22)		
C4a			400, 450	19 (12.7)
C4b			300, 600	14 (9)

morphic in both number and sequence (38). Thus, both the *spa* and *coa* typing methods have been reported to provide a rapid, inexpensive and appropriate method for the genotyping of *S. aureus* strains in epidemiological studies (39). The *spa* method is based on the amplification of the protein A mediating gene (*spa* gene), which generates a staphylococcal strain-specific amplification pattern and can be used for typing of *S. aureus* strains. For example, Luxner et al. could classify clinical isolates of *S. aureus* in 64 groups using *spa* typing (40). In the present study, the *spa* gene length were varied from 1000 to 1450 bp among tested isolates, which are very close to the previously reported range (1150 to 1420 bp) from India (14). Moreover, based on the polymorphism of the *spa* gene, we could clas-

sify isolates into four different types and in this respect our results are similar to another report from Iran (41). S1 (1450 bp) and S2 (1250 bp) types were the most frequent types among all types. In addition, S1 type yielded six restriction patterns after digestion with *HaeII*. Whereas, S2 type yielded three RFLP patterns indicates that S1 type has a greater genetic diversity than type S2. As a result, distinct genetic diversity may exist even between predominant types. Restriction profile analysis of the *spa* gene in all our isolates demonstrated 16 different patterns, which is more than those reported by Mitani et al. in Japan (42). They could determine eight restriction pattern of *spa*, as well as four pattern of the *coa* gene. Beside of *spa* typing, classification based on the *coa* gene has also been considered a simple and accurate method for molecular typing of *S. aureus* (43). In our study, PCR amplification of the *coa* gene resulted in identification of four different types and type C3 (with 700 bp length) as the most frequent type. The polymorphism of this gene is due to repetitions of 3' elements of the *coa* gene in various strains (44). Previously published data from Iran have shown the presence of different *coa* types (45, 46). Talebi-Satlou et al. conducted a similar study on *S. aureus* isolates associated with skin and urinary tract infections in Urmia region of Iran, and showed four *coa* types with 410, 530, 700 and 790 bp length (45). They also reported the *coa* type with 700 bp as the most common type, which is consistent with our results. However, in contrast to their study that determined two RFLP patterns for the dominant *coa* type, we could classified the predominant *coa* type in three subtypes, which indicate great heterogeneity among our isolates. It is noteworthy that, the *coa* type with 700 bp length also was reported as a dominant type in another study from Iran (47). Considering this finding, it maybe suggested that a specific subset of *S. aureus* strain is well- adapted in various parts of human body in different region of Iran. However, expanded genetic analyses are necessary to generate more evidence for this finding.

Overall, we could classify 151 clinical isolates of *S. aureus* in 16 and 9 diverse restriction types based on PCR-PFLP of *spa* and *coa* genes, respectively, which indicate higher discriminatory power of *spa* typing compared to *coa* typing.

Finally, the outcome of our study shows that *spa* typing can be used along with other molecular methods as an appropriate method in epidemiological investigations to control and monitor infections obtained from hospitals and society, in distinguishing *S. aureus* isolates collected from clinical specimens.

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Footnotes

Authors' Contribution: Mohammad Ahangarzadeh Rezaee and Alka Hasani contributed to study concept and design, development of the study, interpretation of data and revision of the manuscript; Seyed Foad Mirkarimi carried out all phenotypic and molecular studies; Vajihe Sheikhalizadeh participated in drafting of the manuscript and statistical analysis; Mohammad Hossein Soroush helped to perform experimental procedures; Babak Abdinia participated in acquisition of data, especially in medical consultation; Mohammad Ahangarzadeh Rezaee supervised the study. All authors read and approved the final manuscript.

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